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(71) Applicant *(for all designated States except US)*: SHELL INTERNATIONALE RESEARCH MAATSCHAPPIJ B.V. [NL/NL]; Carel van Bylandtlaan 30, NL-2596 HR The Hague (NL).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: EDWARDS, Glyn, Alyn [GB/GB]; 25 Blenheim Avenue, Faversham, Kent ME13 8NW (GB).

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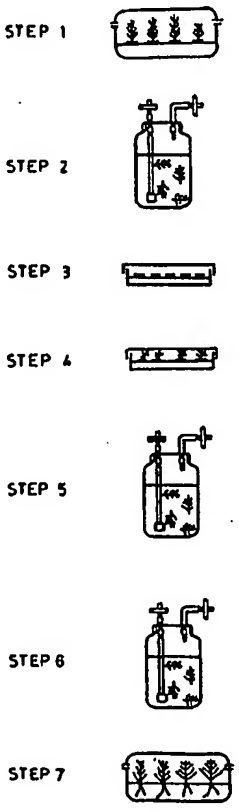
(57) Abstract

A process for producing genetically modified plant material comprising one or more stably incorporated DNA sequence(s) of interest, which comprises subjecting plant material that consists of or comprises shoots, shoot tips or apexes, rooted shoots or seedlings, to *Agrobacterium*-mediated transfer of the DNA sequence(s), the *Agrobacterium*-mediated DNA transfer being carried out in an oxygenated, for example, aerated liquid culture medium in which the plant material is cultivated, the plant material being submerged in the liquid medium. The medium is generally agitated or otherwise moving. The plant material is preferably actively growing in the submerged culture.



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GENETIC MODIFICATION OF PLANT MATERIAL

The present invention relates to a highly efficient process for the genetic modification of plant material.

Methods that enable the introduction and stable incorporation of a DNA sequence of interest into plant material in order to achieve the desired genetic modification are well known and include Agrobacterium-mediated transfer and methods that introduce the DNA directly into cells, for example, electroporation of protoplasts, bombardment of embryos with DNA-coated particles and polyethyleneglycol-mediated gene delivery.

Agrobacterium-mediated genetic transformation methods are generally preferred to direct transformation with DNA for several reasons. The method is relatively fast and simple and not expensive in terms of labour, materials or equipment. In addition, the majority of transformed plants produced using Agrobacterium-mediated transformation contain one or a low number of intact DNA inserts, and the inserted DNA is often stably expressed. Direct DNA transformation methods, such as those described above, frequently result in multiple copies of the DNA insert in the plant cells, and the inserts are often not intact due to rearrangements. Multiple copies of inserted DNA have been associated with instability of expression of the introduced (heterologous) genes. Hence, the advantages of Agrobacterium-mediated over methods which employ direct DNA transformation are such that the latter are usually used only if the plant species is intransigent with respect to Agrobacterium-mediated transformation, for example, because those plant cells that are capable of regeneration into whole plants are recalcitrant to Agrobacterium-mediated transformation.

However, although Agrobacterium-mediated transformation has a number of advantages over direct transformation methods, it would be desirable to increase the efficiency of the method, especially in relation to particular types of plants, for example, woody plants.

The present invention provides a process for producing genetically modified plant material comprising one or more stably incorporated DNA sequence(s) of interest, which process comprises subjecting plant material that consists of or comprises shoots, shoot tips or apexes, rooted shoots or seedlings, to bacterium-mediated, especially Agrobacterium-mediated, transfer of the DNA sequence(s), the DNA transfer being carried out in an oxygenated, for example, aerated liquid culture medium in which the plant material is cultivated, the plant material being submerged in the liquid medium. The medium is generally agitated or otherwise moving. The plant material is preferably actively growing in the submerged culture. The invention includes the use of an Agrobacterium or other bacterial vector. The term "Agrobacterium-mediated transformation" includes transformation using Agrobacterium-related vectors and the term "Agrobacterium" includes other bacterial vectors.

The DNA sequence(s) of interest may be heterologous to the recipient plant, or may be homologous. They should be functional in the recipient plant. Many examples of DNA sequences of interest in the genetic modification of plants are known. For example, the DNA may function to impart to the recipient plant a phenotypic property, e.g. resistance to a herbicide such as glyphosate, to modify the quality or quantity or the chemical components of the plant, to modify rooting ability of vegetative propagules, for example, cuttings,

or to confer reproductive sterility.

The present invention involves introducing an Agrobacterium strain or a related vector to plant material that consists of or comprises shoots, shoot tips or apexes, rooted shoots or seedlings, which plant material is growing in submerged liquid culture and allowing transformation of plant cells within the plant material to occur whilst the cultured plant material continues to grow and develop normally. Preferably the plant material is already growing actively in the submerged liquid culture before the introduction of the Agrobacterium.

The process of the present invention has a number of significant advantages over conventional methods of Agrobacterium-mediated transformation of plant material carried out on semi-solid media. (Gelled media are often called "solid" whether they are in fact solid or semi-solid. Unless specified otherwise, the term "semi-solid medium" as used herein includes both solid and semi-solid forms of gelled media.) One advantage is that the plant material is totally immersed in the liquid medium and hence every part of the plant material is in contact with the Agrobacterium transformation agent, thus enabling more extensive and more effective penetration of the transformation agent into the plant tissues.

A further advantage of the process of the present invention is that the physiological status of the plant tissues facilitates the invasion of the Agrobacterium cells into the tissues, permitting transformation of cells deep within the tissues that would not normally be transformed with high efficiency using existing methods. For example, the plant material may be subjected to minor trauma in the liquid culture. The plant material may be affected by mild hyperhydricity (see below). In both cases entry of the bacterial vector into the plant

tissues will be facilitated.

A further advantage over semi-solid media is that the composition of the liquid medium used according to the present invention is uniform and can be adjusted easily and readily at any time, to establish and maintain optimum conditions for transformation.

For example, by carrying out transformation in a liquid medium according to the present invention, it is possible to establish and to maintain conditions such that growth of the plant material proceeds normally but the growth of the Agrobacterium is restrained, hence preventing overgrowth of the Agrobacterium strain, which can be detrimental to the health of in vitro propagated plant tissues. Furthermore, it is possible to establish and maintain conditions under which the Agrobacterium strain is stimulated to transfer the required segment of DNA (the T-DNA) into the plant cells. Moreover, it is possible to establish and maintain conditions such that, although the growth of the Agrobacterium cells is inhibited, the Agrobacteria remain in a state where transformation can occur, so the transformation can be continued for extended periods of time, for example, for several weeks. This is in comparison to conventional methods using semi-solid media where transformation can be continued for a few days at most.

It is possible both to establish and maintain the required conditions because the transformation medium is liquid. The composition of the medium can be readily monitored and can be adjusted whenever necessary without disturbance to the plant material.

For example, overgrowth of the Agrobacteria may be restrained, transfer of DNA stimulated and the ability of the Agrobacteria to transfer DNA maintained by control of the pH of the liquid medium, for example, by maintaining the pH of the liquid medium at a low level,

for example, within the range of from 4.0 to 5.4, for example, 4.0 to 5.0, preferably 4.5 to 5.4. Alternatively and/or in addition, the liquid medium may comprise a sub-lethal dose of an antibiotic. Any other appropriate agent may be used to achieve the desired effects.

Another advantage results from the facts that the plant material in the liquid culture continues to grow rapidly and normally so transformation can continue for a prolonged period of time, for example, for many weeks, rather than the usual maximum of a few days. Both those plant cells already existing at the time of inoculation with the Agrobacterium strain and the large numbers of cells formed by subsequent plant cell division are available for transformation by the Agrobacterium strain. This results in a high frequency of transformation, with many individual cells of the plant material being transformed independently. For example, the case of Eucalyptus grandis and hybrids thereof, which are normally transformed at low frequency using Agrobacterium-mediated transformation, this effect is particularly marked.

Furthermore, because the plant material in the liquid culture continues to grow and develop normally, each individual transformed cell can continue to divide to give an "island" or sector of genetically identical transformed cells. The process results in the formation of chimeric shoot cultures composed of untransformed cells and many sectors of transformed cells.

The high frequency of transformation and the continued division of the transformed cells result in the plant material containing an exceptionally and unexpectedly high proportion of transformed plant cells. For example, in the case of Eucalyptus grandis and Eucalyptus grandis hybrids transformed according to the

process of the present invention, more than 50% of the tissues in some leaves appeared to be transformed. Hand dissection of the tissues showed that some of the transformed areas consisted of multiple cell types. For example, epidermal, pallisade and mesophyll cells within some single contiguous areas were all found to show marker transgene activity.

A brief description of the accompanying drawings follows. More detailed descriptions are given later.

Figure 1 of the accompanying drawings is a map of a plasmid identified herein as pSCV1, which is used in the production of plasmid pSCV1.6, which may be used in the process of the present invention.

In Figure 1 Amp^R and Gm/Km^R denote antibiotic resistance genes for plasmid selection in bacteria. trfA, trfB, RK2 and Col E1 origins denote baterial replication functions. OD denotes an overdrive (T-DNA transfer enhancer) sequence. Bam H1, Bcl 1, Cla 1 etc denote restriction endonuclease recognition sequences. Map units are given in Kilo base pairs of nucleotide sequence.

Figure 2 is a map showing the T-DNA of the plasmid identified herein as pSCV1.6.

In Figure 2 the orientation of the genes and the region of DNA for transfer to plants are shown. The abbreviations given in Figure 2 have the following meanings: B = Bam H1; Bg = Bgl II; C = Cla 1; E = Eco R1; EV = Eco RV; H = Hind III; K = Kpn 1; P = Pst 1; S = Sac 1; Sm = Sma 1; Sp = Sph 1; X = Xba 1; Xh = Xho 1; OD = Over-drive (T-DNA transfer enhancer)

Figure 3 of the accompanying drawings illustrates, by way of a non-limiting example, steps involved in a typical process for the production of rooted genetically modified plants using transformation in submerged liquid culture, regeneration on a semi-solid medium with no

selection or non-stringent selection followed by stringent selection in submerged liquid culture and propagation of the selected transformed i.e. genetically modified in submerged liquid culture and finally rooting on a semi-solid medium.

According to a process of the present invention, any appropriate bacterial vector may be used to mediate genetic modification of plant material according to a process of the present invention, for example, Agrobacterium tumefaciens or Agrobacterium rhizogenes transformation vectors. Agrobacterium transformation vectors are well known in the art, see for example, EP-0120516-B (US Patent No. 4,940,838). In some such vectors the DNA to be transferred and other DNA sequence(s), for example, encoding a selectable marker, are placed between the right and left borders of a Ti plasmid, thus enabling their transfer. Other systems involve binary vectors. An example of a suitable Agrobacterium tumefaciens strain is the disarmed strain EHA101A containing the binary Ti plasmid pSCV1.6.

According to the present invention, the plant material is cultured under conditions such that it is completely immersed in the liquid medium. The plant material is preferably cultured such that it is growing actively in the submerged culture before the transformation process is started. For transformation, the bacterial transformation vector is simply inoculated into the culture of the plant material and culture is continued, for example, as described below.

The plant material may be allowed to move in the liquid medium, for example, it may be allowed to move freely. For example, the plant material may tumble, for example, tumble freely, in the liquid medium. The tumbling may be vigorous and may be substantially continuous. Alternatively, the movement of the plant

material may be restricted or otherwise impeded. For example, the plant material may be restrained, for example, it may be held by perforated restraining means, for example, in a perforated container, for example, a cage or bag, within the plant growth vessel or within a separate section of the vessel that is in contact with the liquid medium. Minor trauma to the plant material may facilitate entry of the bacterial vector into the plant tissue. Movement of the plant material and/or movement of the liquid culture medium also enhances the distribution of the bacterial vector throughout the medium.

The liquid medium in which the plant material is cultured must comprise sufficient oxygen to support the metabolism of the plant material. It is generally necessary to provide oxygen, usually in the form of air, and/or to illuminate the system such that oxygen is produced by photosynthesis. An appropriate carbon source should also be provided, for example, carbon dioxide or an organic carbon source.

The medium may be agitated by mechanical means, for example, by means of a mechanical device, for example, a paddle or a stirrer, for example, a magnetic stirrer, or the vessel containing the medium may be agitated, for example, shaken, vibrated or rotated. The means used for agitation, for example, shaking or stirring, may oxygenate the medium sufficiently to support the metabolism of the plant material. If not, oxygen, generally in the form of air, may be provided and/or the system may be illuminated such that oxygen is produced by photosynthesis.

The production of oxygen by photosynthesis may totally or in part provide the oxygen required by the culture. The light required for the production of oxygen by photosynthesis will generally cause movement

of the liquid medium by convection.

The liquid medium may be both agitated and oxygenated by passing oxygen or, more usually, air, through the medium. Air circulation techniques, sometimes called "airlift" techniques, are particularly useful for providing simultaneous oxygenation and agitation in the process of the present invention. Liquid medium in an appropriate vessel, for example, a fermentation vessel, for example, a flask, bottle, tank or column may be circulated and oxygenated by the introduction of air through, for example, a gas diffuser. Such vessels are often called "air-lift fermenters". The volume of air and the rate of introduction can easily be adjusted to give the desired degree of agitation to the liquid medium and hence to the plant material when it is allowed to move freely. The passage of a gas through the medium also has the advantage of purging ethylene from the medium. The airlift system appears to be an efficient system for transformation of plant material according to the present invention.

When the plant material is allowed to move freely, agitation of the medium by passing air or another oxygen-containing gas through the medium may be preferable to agitation by mechanical means, for example, stirring, because the shearing forces on the plant material may be lower. However, the shearing forces on the plant material in shaken or stirred systems of the present invention may by reduced by restricting the movement of the plant material, for example, as described in more detail below. In a system where air or another gas is passed through the medium it is possible to restrict the movement of the plant material, so the liquid medium is agitated but the plant material is restrained, should that be desired.

However, shaking or gentle stirring appear to be particularly suitable for transformation systems according to the present invention.

If the passage of the gas is not sufficient to achieve the desired agitation, additional agitation means may be provided, for example, the plant growth vessel may be also be shaken or the contents may be stirred. The vessel may be illuminated to provide further oxygen by photosynthesis.

If it is desired to restrain the plant material in the liquid culture vessel, the plant material may be restrained in a container within the vessel or the vessel may be divided into sections, one or more sections containing plant material. The restraining or dividing means should allow passage of the sufficient liquid medium to allow adequate oxygenation of the plant material. Perforated or mesh materials may be used to construct the restraining means, for example, there may be used a perforated metal or plastics material, a wire mesh or a fabric, for example, muslin. Alternatively, fresh oxygen-containing liquid medium may be passed through a plant growth vessel containing the plant material, either continuously or periodically, the plant material being submerged at all times.

The movement of the plant material may be restricted in any liquid culture system of the present invention, including those where oxygenation is achieved by shaking or stirring the liquid medium, by passing air or another oxygen-containing gas through the liquid medium, by photosynthesis, or by any combination thereof. In the case of stirred cultures, it is particularly preferable to restrain the plant material because it may be damaged by the stirrer if it is allowed to move freely.

It should be noted that after initial inoculation

in the liquid medium the plant material may not become wetted initially because air bubbles may bind to the surfaces. If so, the plant material may float at or near the surface of the medium. A surfactant may be included in the culture medium to assist wetting. Even in the absence of surfactant, however, the surfaces of the plant material will generally become thoroughly wetted within a few days. In an air-lift system, if there is sufficient agitation and they are not restricted, they will generally tumble freely.

The liquid medium used for the transformation may be any medium that is suitable for growth of the chosen plant material. It should generally include sources of carbon and of nitrogen, organic and inorganic salts as required, and appropriate phytohormones and/or plant growth regulators. Suitable growth media are known, and an appropriate medium may be chosen for the particular woody plant to be propagated. Known solid media may be modified by the omission of the gelling or other solidification agent to give a liquid medium. It may also be appropriate to modify levels of one or more of the components of a known medium for use according to the present invention, for example, it may be possible to use reduced levels of phytohormones and/or plant growth regulators compared with levels used in corresponding solid media. The optimum level of any particular component or combinations of components may be determined by conventional methods.

As indicated above, it is advantageous to co-cultivate the plant material and the Agrobacteria at a pH or under other conditions such that growth of the shoot cultures proceeds normally but the growth of the Agrobacterium is inhibited, hence preventing overgrowth of the Agrobacterium strain, which can be detrimental to the health of in vitro propagated plant tissues. Such a

pH is, for example within the range of from 4.0 to 5.4, for example, from 4.5 to 5.4. Because the growth of the Agrobacterium cells is restrained but the Agrobacteria remain in a state where transformation can occur, the transformation can be continued for many weeks. This is in comparison to conventional methods using semi-solid media where transformation can be continued for a few days at most. Furthermore, a low pH of the liquid medium not only prevents Agrobacterium overgrowth, it also stimulates the Agrobacterium strain to transfer the required segment of DNA (the T-DNA) into the plant cells. Other appropriate conditions may be used to achieve the same effect.

Appropriate culture conditions should be used, for example, with regard to temperature and light. It may be preferable to illuminate the culture to provide endogenous oxygenation to supplement or even, in some cases, to replace exogenous oxygenation. If the culture undergoes autotrophic growth under light, the carbohydrate source in the medium may be omitted or reduced. In such cases, the supply of air or another oxygen-containing gas may be supplemented with carbon dioxide. Alternatively, if sufficient oxygen is provided to support the metabolism of the plant material, cultivation may be carried out entirely or partially in the dark.

It is preferable to ensure that all apparatus and all other materials used are sterile to minimise microbial contamination. Apparatus is preferably sterilised before use by autoclaving, and media are sterilised by autoclaving or filtration, where possible, and tissue culture grade materials are preferably used. All joints in the apparatus should be carefully sealed.

Where air or another gas is supplied to a liquid culture vessel, filters are preferably provided in the

inlet and outlet of the air supply to the vessel to maintain sterility within the vessel. The air supply may be passed through a filter, for example, an activated charcoal filter, to remove gaseous and/or volatile contaminants in the air supply. The filters used, particularly exhaust filters, are preferably hydrophobic as there is inevitable evaporation from the apparatus with the potential for condensation in the exhaust air stream. For long-term operation, it may be desirable to incorporate a condenser in the exhaust air stream to avoid build-up of condensation and potential microbial growth that may occur in filters, decreasing flow rates and possibly causing infection of the culture (filter "grow-through" phenomenon).

The plant liquid culture vessel may be of any size and shape suitable for submerged liquid culture. Suitable vessels are well known for "air-lift" systems and for systems where the liquid medium is shaken or stirred. The vessel may be, for example, a flask, bottle, column or tank. The vessel may be of glass, metal or even a synthetic polymer, for example, polypropylene or polycarbonate. If the plant material is to be illuminated for photosynthesis, the vessel should allow the passage of light of the appropriate wavelength.

The liquid medium is introduced into the vessel, is preferably brought to the temperature at which cultivation will be carried out, the inoculum of plant material is added to the medium, and the apparatus is generally sealed. The plant growth vessel and its contents are maintained at an appropriate temperature, for example, from 20 to 30°C, with or without illumination.

In the case when air or another oxygen-containing gas is passed through the liquid medium in which the

plant material is free to move, the supply is preferably adjusted to the maximum flow rate that gives a steady stream of bubbles such that the plant material is submerged and preferably tumbles, and especially tumbles freely, in the medium. The tumbling may be substantially continuous. In the case of systems in which the liquid medium is shaken or stirred, the shaking or stirring should be sufficient to oxygenate the medium while ensuring that the plant material is submerged.

An appropriate medium is chosen for the plant to be transformed, for example, KM medium (without gelling agent) may be used for the transformation of Eucalyptus grandis and hybrids thereof. Similarly, for other plants the gelling agent may be omitted from a solid or semi-solid medium previously used for the propagation of that plant. As indicated above, the pH should preferably be such that overgrowth of the Agrobacteria is inhibited and/or that transfer of the DNA to the plant material is stimulated.

In some cases it may be possible to use levels of phytohormones lower than are conventionally used in solid media, for example, in the propagation of Eucalyptus grandis according to the present invention, good results are obtained with 25% (or even less) of the conventional amount of BAP (6-benzylaminopurine).

Plant material used for transformation may be obtained by micropropagation, or germinated and/or grown in a growth chamber or cabinet, greenhouse or outdoors. Shoots, shoot tips or apexes, rooted shoots and seedlings may be obtained from a cultivar, clone or seed, especially from genetically valuable cultivars, clones or seed, or from genetically manipulated plant material. Shoots, rooted shoots and seedlings used for transformation preferably have one or more nodes, for

example, from two to four nodes. The shoot tip (apical meristem) and axial buds may be removed before inoculation. Alternatively, a shoot tip or apex may be used as the plant material to be transformed.

The transformation process of the present invention has universal applicability, that is to say, it may be applied to plant material of any plants. The plants may be, for example, annual, biennial or perennial plants; they may be mono-cotyledonous or dicotyledonous plants; they may be herbaceous or woody plants. Woody plants are perennial plants that exhibit secondary growth (secondary thickening) of roots and/or aerial stems, which is the result of the formation of wood. Wood is secondary xylem, and is composed of one or more of the following: tracheids, vessels, fibres and rays. Woody plants include forest trees, other trees, shrubs and bushes.

Examples of woody plants that may be transformed by the process of the present invention include, but are not limited to, gymnosperms and dicotyledenous and monocotyledenous angiosperms, for example, as used for wood pulp, for fuel or for timber, for example, Eucalyptus, Pinus, Picea, Acacia, Populus, Betula, Tectona and tropical hardwoods; trees, shrubs and bushes that produce fruit or nuts, for example, apple, citrus, peach, olive, walnut and almond trees, coffee bushes, blackcurrant bushes, and raspberry canes; trees, shrubs and bushes from which other commercially useful products can be obtained, for example, rubber trees and trees and shrubs that produce pharmaceutically useful substances or precursors for pharmaceutically useful substances, for example, yew trees; and ornamental trees and shrubs, for example, trees and shrubs having ornamental flowers, foliage or bark.

Woody species of commercial interest for genetic

modification include Pinus, Picea, Abies, Pseudotsuga, Chaemaecypris, Taxus, Populus, Acacia, Leucaena, Melia, Gmelina, Liquidambar, Betula, Hevea, Tectona, Alnus, Grevillea, Paulonia, Cederla, Coffea, Citrus, Phoenix, Juglans and Elaeis.

As indicated above, eucalyptus is an example of a sclerophyllous woody plant that may be transformed according to the present invention. The sub-genus Eucalyptus Symphyomyrtus contains many commercially useful species, for example, E. grandis, E. globulus, E. nitens, E. dunnii, E. saligna, E. camaldulensis, E. urophylla, E. tereticornis and hybrids thereof. Further commercially important Eucalyptus species are found in the sub-genus Eucalyptus Corymbia and the sub-genus Eucalyptus Monocalyptus, and include E. regnans, E. citriodora, E. fraxinoides E. maculata and hybrids thereof.

Woody plants that appear to perform particularly well in the liquid culture system of the present invention are sclerophyllous species. The definition of sclerophyll is "thick, leathery leaf". This includes true leaves, as in the case of eucalypts and phyllodes as in the case of some Acacias. Species considered as sclerophyllous are generally evergreen and the sclerophyllous habit is generally associated with poor nutrient availability and often with drought tolerance. Examples of sclerophyllous genera are Rhododendron, Azalea and Kalmia (Ericaceae); Olea (Oleaceae); many Australian Acacias (Fabaceae); and eucalypts (Myrtaceae).

However, it is not only sclerophyllous species that perform well in the liquid culture system of the present invention. Malus (apple), Pyrus, Prunus and Rosa (Rosaceae), Forsythia and Syringa (Oleaceae) are further examples of woody plant genera that may be

transformed using the liquid culture system of the present invention.

It is generally advantageous to use, as starting material for genetic manipulation, plant cells or tissues that are genetically uniform, for example, cells or tissue derived from homozygous seed or clonal material that is vegetatively derived, directly or indirectly, from vegetative tissues of plants that have been selected, or are selectable, for favourable characteristics.

However, in some cases, for example, woody plants, especially trees, for example, Eucalyptus, a desired characteristic can only be assessed in a mature plant, but clonal material obtained from mature plants is often difficult to modify genetically and/or recalcitrant to shoot induction. This is particularly the case in woody plants, especially trees, for example, Eucalyptus and the other species of trees mentioned above.

A particular embodiment of the present invention enables cells and tissue derived via vegetative propagation i.e. clonal material, especially clonal material from plants exhibiting superior phenotypic properties, to be modified genetically, selected and regenerated into viable plants, rapidly and in high yields.

The cell or tissue material, especially cells and tissue derived via vegetative propagation i.e. clonal material, especially clonal material from plants exhibiting superior phenotypic properties, may be obtained directly from a plant grown in the field or a greenhouse. It may be used in non-sterile form, i.e. without the use of an intervening micropropagation step, for the introduction of heterologous (or homologous) gene(s). Alternatively, the cells or tissue may be derived indirectly from selected plants that is to say,

the cells or tissue taken from the selected plant is subjected to micropropagation before genetic manipulation.

In the case of clonal material, the starting material may be obtained from any plant of interest. The plant may be a mature tree, for example, Eucalyptus. In the case of Eucalyptus, it may be obtained, for example, from a member of the sub-genus Eucalyptus Symphyomyrtus for example, E. grandis, E. globulus, E. nitens, E. dunnii, E. saligna, E. camaldulensis, E. urophylla, E. tereticornis and hybrids thereof, or from a member of the sub-genus Eucalyptus Corymbia or the sub-genus Eucalyptus Monocalyptus, for example, E. regnans, E. citriodora, E. fraxinoides E. maculata and hybrids thereof.

The cells or tissue used as starting material for genetic modification according to the present invention may be derived from seedlings, especially young seedlings. The process of the present invention is particularly useful for the genetic modification of cells and tissue obtained from seedlings within the sub-genus Eucalyptus Symphyomyrtus, for example, seedlings of E. grandis, E. globulus, E. nitens, E. dunnii, E. saligna, E. camaldulensis, E. urophylla, E. tereticornis and hybrids thereof, or seedlings of a member of the sub-genus Eucalyptus Corymbia or the sub-genus Eucalyptus Monocalyptus, for example, seedlings of E. regnans, E. citriodora, E. fraxinoides E. maculata and hybrids thereof.

Any appropriate Agrobacterium or related vector may be used to mediate genetic modification of the plant material, for example, Agrobacterium tumefaciens or Agrobacterium rhizogenes. The Agrobacterium tumefaciens strain used to transform E. grandis clones, E. grandis/E. camaldulensis hybrid clones and E. saligna/E.

tereticornis hybrid clones as described in the Examples is the disarmed strain EHA101A containing the binary Ti plasmid pSCV1.6. Figures 1 and 2 of the accompanying drawings are maps relating to plasmid pSCV1.6. Strain EHA101A may be used for the transformation of other Eucalyptus and also of any other plants. Examples of binary Agrobacterium-Ti plasmid vector systems have been fully described elsewhere, e.g. in EP-A-0120516.

Microbial contamination is a potential problem in tissue culture systems in general. It may be a particular problem when the starting material has been obtained from plants grown under non-sterile conditions, for example, in a glasshouse or outdoors, even though surface sterilisation or disinfection is carried out according to conventional methods. Even when the starting material has been produced under sterile conditions there may be microbial contamination from other sources. It also arises in connection with genetically manipulated plant material obtained using Agrobacterium-mediated transfer. Such material may present particular problems for subsequent propagation, particularly micropropagation, because of the inevitable contamination with the Agrobacteria themselves. Plants contaminated with Agrobacterium may also be unsuitable for commercialisation because of regulatory considerations.

According to the present invention, before and/or after transformation using Agrobacterium, the genetically modified plant material may be grown in liquid culture under various specific conditions, for shoot induction, for selection and/or for propagation. In any or all of those steps, the plant material, for example, shoots, shoot tips or apexes, rooted shoots or seedlings may be cultivated in an oxygenated liquid medium that comprises an antibiotic, for example,

augmentin, the plant material being submerged in the liquid medium. The fact that the plant material is totally immersed in the liquid medium results in more efficient penetration of antibiotic into the tissues of the plant material than occurs on solid media, and hence microbial contamination may be reduced or even removed in a simple and effective manner.

After transformation, it is necessary to select the plant material that has been transformed and to convert the transformed plant material, which may be transformed cells or transformed sectors of chimeras, into viable transformed plants. The transformed plant material may be converted by organogenesis or somatic embryogenesis into plants. The basic techniques of organogenesis and somatic embryogenesis are well known, see for example, George EF (1993, 1996) Plant propagation by tissue culture. Vol 1 (2nd Ed.) and Vol 2, Exergetics Limited. Various preferred methods have been described for particular plants. Such methods generally involve induction of shoot formation and selection of transformed shoots, then regeneration of the transformed shoots into roooted shoots and hence plants.

Induction of shoot formation is conventionally carried out on semi-solid media and such methods may be used after transformation according to the present invention. Shoot induction in a liquid medium may be carried out as an alternative to or on addition to shoot induction on semi-solid media.

Whether using a semi-solid or a liquid medium, the shoot inducing agent should be capable of inducing, preferably at high frequency, the formation of buds that are capable of further development. Such agents are generally cytokinins. The suitability of a particular agent for any particular plant starting material and appropriate concentrations of the selected agent and

regimes for its use may be determined by routine methods. The agent is incorporated in the culture medium used for shoot induction, for example, in the liquid or semi-solid medium used for the selection of transformed shoots. Two or more agents may be used.

The cytokinin BAP is a suitable shoot induction agent for many plants, for example, apples and poplar. A further example of a shoot inducing agent for use according to the present invention is the substituted phenylurea N-(2-chloro-4-pyridyl)-N'-phenyl-urea, often known as 4-PU or CPPU. CPPU has been found to induce bud formation in Eucalyptus and other plants at high frequency and, unlike some other phytohormones and plant growth factors, a further effect is that the buds produced are capable of further development into shoots. Other substituted phenylureas may be used instead of or in addition to CPPU for the selected plant material, for example, Eucalyptus, provided they are capable of inducing, preferably at high frequency, the formation of buds that are capable of further development, see WO96/25504.

The culture medium used for induction of shoot formation may contain glutamate and/or ascorbic acid, in order to promote regeneration of shoots at high efficiency. The starting pH may be 5.0-5.6. The induction of shoot formation is generally carried out by culture on a semi-solid medium or using another static culture medium. Examples of media suitable for use in the process of the present invention for inducing shoot formation, for selection of transformed cells and tissue and for multiplication and inducing root formation of genetically modified Eucalyptus are given herein. Suitable media for many other plants are known or may be determined by routine methods.

A further aspect of the present invention relates

to the selection of plant material that has been modified genetically by the stable incorporation of one or more DNA sequences of interest according to the transformation process of the present inventiion and that has a selectable characteristic, property or attribute that enables selection.

A selectable characteristic, property or attribute may require a selective agent to enable selection. Such a characteristic, property or attribute is often called a selectable marker. Selectable markers are well known and include, for example, genes that confer resistance to a selective agent, for example, an antibiotic or herbicide, or to another selective agent. Selection is generally carried out by growing the material that has been subjected to transformation on a medium that contains the selective agent, for example, the antibiotic, herbicide or other selective agent.

Conventionally, selection is carried out on solid media i.e. solid or semi-solid gelled media. The process is generally slow and laborious. In the case of some plant species and/or when using some selectable marker genes and the appropriate selective agent, the process may be extremely slow, labour intensive and expensive.

The selection process involves culturing explants that have previously been transformed using either Agrobacterium-mediated or direct transformation techniques and in which a proportion of the cells contain introduced genes, on a medium containing an appropriate selective agent. The conditions and concentration of the selective agent are generally chosen such that there is inhibition of growth and/or development of untransformed cells and tissues. Cells containing a DNA insert containing the appropriate selectable marker gene continue to grow and/or develop and can therefore be

identified. Alter-natively, transformed and untransformed cells may be differentiated on the basis of different phenotype when in contact with the selective agent, for example, on the basis of the amount of pigment production or altered growth rate.

Optimally, the conditions and concentration of selective agent are chosen such that the transformed and untransformed cells and tissues may be differentiated after a short period of culture. In practice, however, the presence of the selective agent in sufficient concentrations to differentiate between transformed and untransformed cells and tissues may also slow or delay the growth and/or development of the transformed cells and tissues when compared to growth and development under non-selective conditions. That may be due to one or more factors, including the selectable marker gene giving rise to sub-optimal levels of resistance or tolerance to the selective agent, or to indirect effects.

Indirect effects may include the selective agent affecting the growth and development of untransformed cells or tissues surrounding transformed cells or tissues. The normal growth or development of untransformed cells or tissues may be required for these surrounding cells tissues to make a contribution to the normal growth and development of the transformed tissues (nurse effects). Indirect effects may also be due to release of toxic or inhibitory substances from the untransformed cells or tissues, which then have a deleterious effect on the transformed cells and tissues.

For example, when selecting on 35 mg l^{-1} G418 (geneticin), it can take more than six months to produce shoots of transgenic Eucalyptus grandis (or hybrids thereof) from leaf explants derived from field-grown

clones on solid media after trans-formation with a disarmed Agrobacterium strain containing a plant-expressed NPTII gene. In contrast, the time taken for the production of untransformed shoots on similar culture medium lacking G418 is about six to nine weeks. Hence although the presence of the selective agent enables transgenic shoots to be produced and inhibits the production of non-transgenic shoots, the presence of the selective agent may significantly slow the process by which the transgenic shoots are produced.

Furthermore, the quality of plants produced in tissue culture after extended periods of culture may also be poor, particularly if their production requires extended periods of culture of callus tissues. There are many examples in the literature where such extended periods of growth in tissue culture can result in the production of abnormal plants due to the phenomenon of somaclonal variation. Any reduction in the period of time spent in tissue culture should therefore reduce the risk of producing transgenic plants that are abnormal.

By way of example, methods as described above for selecting transformed material and producing rooted shoots of each genetically modified line (i.e. all plants derived from a single transformed cell) of a field-derived (clonal) Eucalyptus grandis (or a hybrid thereof) suitable for weaning and further growth in the greenhouse or field takes about 11 months to produce 100 shoots.

The present invention provides a process for selecting genetically modified plant material produced according to the present invention, which plant material comprises one or more stably incorporated DNA sequences of interest and has a selectable property, attribute or characteristic that enables selection using a selective agent, and the resulting genetically modified shoots are

selected on a semi-solid medium that comprises the selective agent and/or in an oxygenated liquid culture medium comprises the selective agent, the shoots being submerged in the liquid medium. The liquid medium is generally agitated or otherwise moving. The plant material is generally shoots, shoot tips or apexes, rooted shoots or seedlings.

Selection in liquid culture may be carried as an alternative to or in addition to selection on semi-solid media, in which case it may be carried out before and/or after selection on semi-solid media.

Characteristics, properties and attributes suitable for use in selection processes are well known. For example, the NPTII gene may be used as a marker gene, with resistance to a phytotoxic selective agent conferred by that gene, for example, resistance to paromomycin, G-418 (also known as geneticin) neomycin or kanamycin used as the characteristic for selection of transformed cells or tissue. Any other DNA sequence that confers the same or similar resistance may be used as the selectable marker.

Selection in liquid culture is carried out as described above for transformation in liquid culture except that means for selection are present in the liquid culture medium. The plant material may be allowed to move in the liquid medium, for example, they may be allowed to move freely, for example, to tumble, for example, tumble freely, in the liquid medium. Alternatively, their movement may be restricted or otherwise impeded.

The total immersion of the plant material in a solution of the selective agent results in more effective and more extensive penetration of the selective agent into the plant tissues and hence enables effective selection of the genetically modified plant

material.

Selection in a submerged liquid culture may be the only selection method used, or it may be used in addition to selection on semi-solid media. For any plant that is particularly susceptible to hyperhydricity it may be preferable to use only semi-solid media. However, for those plants that are less susceptible to the effects of hyperhydricity, selection wholly or partially in submerged liquid culture is generally advantageous for the reasons given above.

Various permutations and combinations of shoot induction and selection regimes on semi-solid media and in submerged liquid cultures may be used. If desired, a selection process of the present invention, that is to say, selection in a liquid medium, may be incorporated in a protocol in which a selection step on semi-solid (gelled) medium is carried out before and/or after selection in the liquid medium. If used before selection in a liquid culture, selection on a semi-solid medium may be less stringent than would be required if the selection step on the semi-solid medium were the only selection step. A reduction in stringency reduces adverse effects on growth and development that may be caused by stringent selection conditions.

A particular advantage of selection in liquid culture is that the selected material can be already in the form of rooted or readily rootable shoots, rather than as a primordial mass, as in conventional selection methods.

There is the further advantage that shoots or rooted shoots resulting from the selection in the oxygenated liquid medium are of high quality, showing particularly uniform growth. This is particularly useful for the further cultivation of the selected plant material.

The most dramatic advantages over conventional selection methods are the speed at which genetically modified plant material can be selected, and the reduction in costs, including both labour and materials.

The selection process of the present invention has universal applicability, that is to say, it may be applied to selection of genetically manipulated plant material, for example, shoots, shoot tips or apexes, rooted shoots and seedlings of any plants.

As an alternative to using a selectable marker gene and a selective agent, transgenic plants may be selected using methods that are based on the detection of an introduced nucleic acid sequence (transgene), for example using PCR, or of the product of an introduced nucleic acid sequence, for example, an enzyme. Such methods do not require the use of a conventional selective agent.

However, in methods of transformation used previously, the events of transformation and regeneration that give rise to transgenic plants are relatively rare, so large populations of shoots or plants must be screened in order to detect those plants that have been transformed. The rareness of transformation events and the time-consuming, labour-intensive and, particularly in the case of PCR, expensive nature of the detection of a transgene have resulted in this technique being used much less than selecting for a selectable marker gene using a selective agent.

In contrast to rarity of transformation events using conventional methods, the high efficiency of the transformation process of the present invention is such that detection of a transgene or its product can be regarded as a viable alternative to the more conventional selection process. Accordingly, after

transformation according to the process of the present invention, the resulting plant material may be regenerated, for example, on a semi-solid medium, for example, as described above. A transgene or its product may then be identified. This selection technique may be used for selection of transformed material derived from shoots, shoot tips or apexes, rooted shoots or seedlings.

For example, an introduced nucleic acid sequence may be detected directly, for example, using PCR or another amplification technique. The product of an introduced nucleic acid sequence may be an enzyme, which may be detected in an appropriate manner. For example, the ß-glucuronidase gene is a suitable transgene, the product of which, ß-glucuronidase is readily identifiable by standard assays. In a further embodiment, the product of an introduced nucleic acid sequence may itself be directly detectable, for example, the product of a transgene may be a pigment or a fluorescent moiety, or another detectable phenotypic characteristic. Transgene products that are directly detectable in situ without the need for a destructive assay or the use of molecular analysis such as PCR are particularly useful. An example of a gene that produces an identifiable product is the green fluorescent protein (GFP) gene, which allows identification of the gene product in situ (Reichel et al. (1996)).

Whether shoot induction and/or selection is carried out wholly or in part in submerged liquid culture or whether both shoot induction and selection are carried out on semi-solid material, it is advantageous to grow on i.e. to propagate the transformed plant material using the submerged liquid culture system described above.

Should signs of hyperhydricity (vitrification) be

observed during use of the liquid culture technique of the present invention, such signs are generally readily reversed during culture on a semi-solid medium. In general, symptoms of hyperhydricity if they should occur, are mild and are only apparent on well expanded, older leaves. Younger material (i.e. at the shoot tips and apical nodes) does not generally show any significant signs of hyperhydricity. Such signs of mild hyperhydricity at the transformation stage will generally be readily reversed during the stage of regeneration/shoot induction on a semi-solid medium. However, should such signs become serious, the plant material may simply be removed from the liquid culture medium and regenerated on semi-solid medium. Although one of the advantages of the transformation process of the present invention is that it may be carried out for weeks rather than days as when using conventional methods on semi-solid media, the high efficiency of transformation according to the process of the present invention affords advantages over the conventional methods even if transformation is carried out only for a few days. Similarly, if selection and/or propagation is carried out in liquid culture, mild hyperhydricity may be reversed by conventional cultivation on semi-solid media.

When shoots of a woody plant comprising an apical meristem, for example, Eucalyptus grandis and hybrids thereof, are propagated according to the air-lift system of the present invention with the shoots tumbling freely in the liquid medium, the effects of correlative inhibition on axillary shoot meristems appear to be almost completely abolished. All the branch systems originating from existing nodes present at time of inoculation contain tertiary branches. Nodes of decreasing age resulting from extension growth from the

apex of the inoculated shoot possess branch systems of decreasing complexity. The shoot tips of the original stems, the primary branches and the secondary branches all yield shoot tips that are well elongated, possess thick and robust stems and two or more well spaced nodes. There is remarkable degree of uniformity between the harvested shoots, regardless of the number of nodes that the source branches possessed at harvesting. Almost all of the tertiary branches consist of a shoot tip and at least two expanding leaves.

When shoots are propagated in a submerged liquid culture system of the present invention where their movement is restricted, for example, by being held in a perforated container, for example, a cage or bag, within the plant growth vessel, the resulting shoots display substantially the same characteristics as described above for shoots that are able to move freely. They display a high degree of uniformity and are of good quality. Furthermore, the multiplication rates are generally as high as those for the freely moving shoots. It appears that a high shoot inoculation density may lead to a high multiplication rate.

Shoots harvested from the plant growth vessels root well and, for example in the case of E. grandis, rooting efficiency may be better than that resulting from methods of propagation that utilise solid media. In the case of Acacia mangium, the resulting shoots can be rooted in compost and transferred directly to the greenhouse without the need for rooting on solid media. This is a most surprising advantage, which is very important commercially. The quality of the Rhododendron and eucalyptus shoots and their good rooting efficiency indicates that they, too, may be rooted directly in compost and transferred to the greenhouse without an intermediate rooting stage in semi-solid media. Indeed,

the good quality of the material obtained according to the process of the present invention suggests that direct rooting in compost and transfer to the greenhouse may be possible with other genera in addition to Acacia and Eucalyptus.

In summary, the propagation in submerged liquid culture of plant material that has been genetically modified according to a process of the present invention yields an abundance of green, healthy, highly uniform shoots capable of rooting or further propagation. In many cases the shoots are sufficiently elongated that they can be rooted directly without further specific elongation steps. In some cases the shoots can even be rooted directly in compost and transferred to the greenhouse without an intermediate stage of rooting on semi-solid media.

The extension rates of new shoots propagated using the liquid culture system appears to be unusually uniform. For commercial micropropagation, uniformity of product is extremely important. Any developmental heterogeneity of shoots set for rooting is often amplified during subsequent development of the plants. Such heterogeneity is usual in woody plants, resulting in the need for grading of products and possible interruptions of supply.

There are also major cost advantages compared to propagation systems that use solid media, including savings in manual labour (or the high capital costs of automating processes using solid media), disposables and time. For instance, to produce 50 shoots of E. grandis or a hybrid thereof from a single starting shoot using typical solid media protocol requires three subculture steps (and associated media preparation and use of disposable culture dishes) and takes three months. This compares to a single culture step and one month using

the system of the present invention.

Thus, transformation of plant material in submerged liquid culture according to the process of the present invention followed by further steps in submerged liquid culture is highly attractive commercially. The efficiency of transformation is higher than in conventional systems. Selection in submerged liquid culture has advantages over selection according to conventional methods, as has propagation in liquid culture. While an appropriate protocol for transformation, selection and propagation will be chosen for each type of plant material individually, it is generally advantageous to carry out as many steps as possible in submerged liquid culture. The yields obtained are generally higher than those with gelled systems, and are often much higher. The high quality of the product, both in the general health of the product and its remarkable uniformity, is a particular commercial attraction. For some species there are further specific advantages, as described above.

Figure 3 of the accompanying drawings illustrates, by way of non-limiting examples, steps involved in a typical process for the production of rooted genetically modified plants using transformation in liquid culture according to a process of the present invention. Liquid culture is carried out using a submerged "air-lift" system in which the plant material is allowed to move freely. The time scale of the process and the media used are described below by way of example for the production of genetically modified Eucalyptus grandis hybrid plants. It will be appreciated that using the protocol illustrated in Figure 3, the time scale and media of choice will depend on the nature of the plant material to be modified genetically. Furthermore, the steps illustrated may be carried out in a different

order, steps may be omitted and/or other steps added. Any of the other liquid systems of the present invention may be substituted for the "air-lift" system, with similar results.

In Step 1, explants for example of Eucalyptus grandis hybrids are disinfected and established in micropropagation cultures on a semi-solid medium.

In Step 2, the explants are transformed with a DNA sequence of interest using Agrobacterium-mediated transformation in submerged liquid culture. In addition to the DNA sequence of interest, the transformed material will also contain a selectable marker gene, for example, the NTPII gene. The plant material and the Agrobacteria may be cocultivated for a prolonged period, for example, for several weeks. Additionally, several rounds of cocultivation may be performed with fresh plant growth medium being added and/or additional inoculations of Agrobacterium tumefaciens cells.

In Step 3 the transformed plant material is harvested and transferred onto a semi-solid regeneration medium, for example, a clonal regeneration medium containing a shoot inducing agent. For Eucalyptus it is particularly advantageous to use CPPU to induce shoot formation.

In Step 4, shoots are regenerated on semi-solid medium containing a shoot inducing agent. For Eucalyptus, it is again advantageous to include CPPU in the solid medium to induce shoot formation. The medium preferably contains an antibiotic to prevent the growth of the Agrobacterium, for example, augmentin. The medium may also contain the appropriate selective agent. For selecting transformants containing the NTPII gene the selective agent is, for example, paromomycin, G418, kanamycin or neomycin. However, if selection is carried out at this stage, it is preferably "soft" i.e. non-

stringent selection.

For Eucalyptus the solid medium is, for example, clonal shoot induction medium with CPPU and augmentin, and optionally containing a suitable selective agent, for example, G418 or paromomycin. For Eucalytpus, subculture is carried out every three weeks until shoots appear. Shoots generally appear after about 3-8 weeks and are allowed to continue development up to a total of about 9 weeks; this process may be delayed in the presence of a selective agent.

In Step 5 selection of transformants and shoot elongation is carried out simultaneously in submerged liquid culture using an "air-lift" system in which the shoots are allowed to move freely, as described above. The liquid medium contains an appropriate selective agent, for example, paromomycin for the NPTII gene and, preferably also an antibiotic, for example, augmentin. In this case selection and shoot elongation takes less than about 18 days. The non-transformed material rapidly becomes brown, for example, the first signs show within about 4 to 8 days. Shoots that are totally transformed, in contrast, are healthy and green and the shoots rapidly elongate. Shoots that are composed of both transformed and non-transformed tissues (chimaeric shoots) are easily differentiated from shoots that are composed entirely from transformed cells either by the presence of brown sectors or by partial browning over the entire surface of the shoot. The totally transformed shoots are highly uniform and of very good quality. After about 10 to 18 days, high quality transformants are available for micropropagation. The selective agent is able to penetrate the plant tissues more effectively and more extensively in the submerged liquid culture according to the process of the present invention than is possible when the selective agent is

present in a solid medium.

Micropropagation of the shooted transformants is carried out in Step 6 using a submerged "air-lift" liquid culture according to the present invention in which the shoots are allowed to move freely. In such a system, for example, using liquid KM medium, more than 100 shoots are obtained in about 6 weeks.

The final step, Step 7, involves rooting the shoots on semi-solid medium, for example, KM medium with IBA. It takes about 4 weeks.

Genetically modified plant material, including genetically modified plants, obtained using a transformation process of the present invention is itself part of the present invention, as are products obtained from such genetically modified plant material.

The present invention also provides genetically modified plants obtained from the plant material transformed and optionally selected and/or propagated according to the present invention.

Such plants may themselves be micropropagated according to the micropropagation process described herein. As indicated above, this is particularly useful for the clonal propagation of mature trees, for example, eucalypts.

The following non-limiting Examples illustrate the present invention.

EXAMPLE

Unless specified otherwise the media, plant materials, temperature, airflow and lighting conditions described below were used in the following Example.

Media

Solid KM media for micropropagation of E. grandis & E grandis hybrids

Phytagel	3 g/l
Sucrose	10 g/l
10 X Macronutrient solution[1]	100 ml/l
$MgSO_4.7H_2O$	0.925 g/l
NH_4NO_3	0.825 g/l
Murashige and Skoog (1962)[2] basal salt micronutrient stock solution (Sigma M0529)	50 ml/l
1000 X Murashige and Skoog (1962) vitamin solution (Sigma M03900)	0.5ml/l
BAP (6-benzylaminopurine)	0.04 mg/l

Adjust pH 5.6. with KOH and autoclave at 121°C for 20 minutes.

[1]10 X macronutrient solution contains 2.2 g/l $CaCl_2.2H_2O$, 0.85 g/l KH_2PO_4 and 1.9 g/l KNO_3

[2]Murashige T; Skoog F: (1962) A revised medium for rapid growth and assay with Tobacco tissue cultures. Physiol. Plant., 15 473-497.

Liquid KM media for micropropagation of E. grandis & E. grandis hybrids

Ingredients: as for the solid KM medium, except that

(i) the Phytagel is omitted and

(ii) 0.01 mg/l BAP are used instead of 0.04 mg/l BAP that is used in the solid medium, and

(iii) the medium is filter sterilised through a 0.2 µm filter apparatus.

Half-strength KM medium for rooting of shoots of E. grandis and E. grandis hybrids

This medium contains half the macronutrient,

micronutrient, $MgSO_4.7H_2O$ and NH_4NO_3 content of the basic solid KM medium. BAP is omitted and replaced with 0.2 mg/l IBA (indoyl-3-butyric acid). The pH is adjusted to pH 5.6 and the medium is autoclaved at 121°C for 20 minutes as described above for the full strength basic KM medium. The IBA was filter-sterilised and added after autoclaving.

Where applicable, 1.0 g/l activated charcoal is added prior to sterilisation.

Temperature, airflow and lighting conditions

Liquid micropropagation, micropropagation and rooting on semi-solid media were conducted at 22°C with a 16 hour photoperiod (50-70 µmol m^{-2} s^{-1}, supplied by fluorescent lamps). Unless specified otherwise, airflow rates were in the range of from 0.3-0.7 litre per minute per litre of liquid medium.

Plant material used for inoculation

All plant materials used for inoculation were previously micropropagated on semi-solid media.

E. grandis/E. camaldulensis hybrid clone 11/25 was supplied by the South African Forestry Research Institute, PO Box 727, Pretoria 0001, Republic of South Africa (now FORESTEK, Private Bag X11227, Nelspruit 1200, South Africa). Stock plants were obtained by felling mature trees and harvesting cuttings from new growth arising from epicormic buds in the stump. Cuttings were rooted using routine silvicultural techniques and subsequently potted into 10 litre pots and maintained in the glasshouse as hedged stockplants.

Micropropagated cultures were initiated from the stockplants onto semi-solid medium by placing nodal sections from stockplants or coppice shoots in 250 ml plastic-capped polystyrene specimen jars (Medfor

Products) containing 200 ml sterilising solution (25% Milton) and 0.05% v/v Tween 20 (Sigma) and agitating gently for 15 minutes. The nodal sections were given two rinses in 200 ml sterile distilled water. On each rinse the explants were agitated for one minute. Approximately 2 mm of tissue was removed from each end of the stem using a sterile scalpel, in order to remove bleach-damaged tissue. The explants were then transferred to suitable containers (i.e.Sigma Phytatrays, clear polystyrene 114 x 86 x 65 mm) containing semi-solid KM medium; the base of the explants being inserted a few millimeters into the medium and incubated under the conditions as previously described. After several weeks incubation, axillary shoots were harvested and transferred to fresh semi-solid KM medium for multiplication. The micropropagated cultures were subcultured to fresh KM medium at 4-weekly intervals.

Air-lift fermenter apparatus and operation

The apparatus consisted of a compressed air supply fed via a combined pressure regulator and gauge. A pressure release valve (NUPRO SS-6C-MM-10), activated when the pressure reaches approximately 10 p.s.i. (approximately 67,000 Pa) was fixed into the line downstream of the regulator. The airstream was passed through an activated charcoal gas filter (Whatman Carbon Cap 75) and humidified by passage through distilled water using a gas diffuser (Pyrex no. 2) and a 2l Nalgene square vessel (cat. no. 2015-2000) with a Nalgene venting/filling closure. The deodorised and humidified air stream was used to supply an air-lift fermenter. The airstream was sterilised by passage through a gas filter (Whatman Hepa-Vent 0.3mm pore size) before being fed into the air-lift fermenter. The air-lift fermenters comprised a 2l culture vessel (Nalgene

cat. no. 2015-2000) with a Nalgene venting/filling closure). The airstream was passed through one of the ports such that the air passed into a gas diffusion tube (Pyrex no. 2) inside the vessel. The diffuser was sited so that it was approximately 0.5cm from the base of the assembled culture vessel. The exhaust was vented to the atmosphere via a gas filter (Whatman Hepa-Vent). Silicone tubing (tissue culture grade, Merck) was used throughout the apparatus downstream of the activated charcoal filter. The assembled air-lift fermenter vessel was autoclaved at 121°C for 20 minutes prior to being filled with 2l of sterile liquid KM medium as described above. The fermenter was connected to the air supplies and equilibrated for 1 hour prior to inoculation with plant material. The fermenter was operated under the conditions previously described using an air-flow rate of approximately 0.9 litres/minute.

Micropropagated shoots of E. grandis or E. grandis hybrids having apical meristems were used as starting material. Each shoot possessed 2-4 nodes and a shoot tip. The shoots had previously been serially subcultured on basic solid KM medium containing 0.04 mg/l BAP as described above.

Disarmed Agrobacterium strain

The construction of A. tumefaciens strain EHA101 has been described by Hood et al., 1986. The strain consists of a derivative of the of nopaline A. tumefaciens strain C58 in which the native Ti plasmid has been removed and replaced with the disarmed Ti plasmid pEHA101 in which the wild-type T-DNA (encoding genes for opine synthesis and phytohormone biosynthesis) has been deleted from the Ti plasmid and replaced with a bacterially-expressed kanamycin/neomycin resistance gene. The disarmed plasmid pEHA101 is a derivative of

the wild-type Ti plasmid pTiBo542 isolated from A. tumefaciens strain Bo542 (AT4) which is a L,L-succinamopine producing strain (Hood et al., 1986). Strain EHA101A is a chloramphenicol resistant mutant of strain EHA101 which was isolated by Olszwelski et al., 1988.

Binary vector construct

The Agrobacterium strain used in the transformation contained the binary Ti plasmid pSCV1.6, the construction of which has been described in Patent Specification WO 96/25504:

The strain used in the transformation also contains the binary Ti plasmid pSCV1.6, which is a derivative of pSCV1. Genetic manipulations involving these plasmids were performed using standard techniques (Sambrook et al., 1989). The component parts of pSCV1 are derived from the following (gram-negative) plasmids: the sequence used for the right DNA border and overdrive sequence was synthesised using sequence information from from the TL right border of the octopine Ti plasmid pTiA6 (Peralta et al., 1986). The left border was synthesised using sequence information from the TL of the same Ti-plasmid (Simpson et al., 1982) and is identical to the TL left border of the octopine plasmid pTiACH5 (Holsters et al., 1983). Octopine-type border sequences were used as these have been shown to promote more efficient tumour formation when used in conjunction with the hypervirulent strain EHA101 (Hood et al., 1986). The 97bp polylinker containing restriction enzyme sites for cloning genes into the T-DNA was derived from pUC19 (Yannish-Perron et al., 1985). The high copy number origin of replication which is active in E. coli cells but not Agro-bacterium cells was derived from pUC19 (Yannish-Perron et al., 1985). The

origin of replication of pUC 19 which was itself originally derived from the plasmid ColE1, a plasmid isolated from E. coli. The actual pUC sequence used has been extensively deleted to remove some non-functional (superfluous) DNA sequences. The low copy number origin of replication which is active in both E. coli cells and Agrobacterium cells was derived from the the broad host-range Inc P plasmid RK2. The origin used is a minimal 4.3kb origin which was constructed by deleting most of the non-functional sequences originally present in the wild-type RK2 plasmid (Thomas et al., 1980). The minimal origin therefore contains only two genes (trf A and trf B) and associated non-coding sequences needed for replication in bacteria. The bacterially-expressed gentamicin/kanamycin resistance gene was derived from the plasmid pSa (Edwards, 1988) and is probably an aminoglycoside acetylase (Valantine and Kato, 1989). It has no apparent homology to the neomycin phosphotransferase II coding region (Edwards, 1988). The bacterially-expressed ampicillin/carbenicillin resistance (ß-lactamase, bla) gene was cloned from pUC19 (Yannish-Perron et al., 1985). A genetic and restriction map of pSCV1 is shown in Figure 3.

In Figure 1 Amp^R and Gm/Km^R denote antibiotic resistance genes for plasmid selection in bacteria. trfA, trfB, RK2 and Col E1 origins denote baterial replication functions. OD denotes an overdrive (T-DNA transfer enhancer) sequence. Bam H1, Bcl 1, Cla 1 etc denote restriction endonuclease recognition sequences. Map units are given in Kilo base pairs of nucleotide sequence.

pSCV1.6 is a derivative of pSCV1, into which a plant-expressed ß-glucuronidase (GUS) gene and a plant-expressed kanamycin resistance gene were cloned between the T-DNA borders. The CaMV-NPTII was derived from the

construct of Fromm et al., 1986. However, it has been reported that several of the most common NPTII genes used in plant genetic-manipulation encode a mutant enzyme that has a reduced ability to detoxify kanamycin (Yenofsky et al., 1990). The mutation involves a single base change, resulting in the replacement of a glutamic acid residue by an aspartic acid at the active site of the neomycin phosphotransferase (NPTII) enzyme (originally isolated from the bacterial transposon Tn5). While the stability of the mRNA and the protein appeared unaffected by the mutation, the enzyme activity towards kanamycin is significantly reduced. The presence of the mutation in a gene can be identified by checking for the loss of a site for the restriction endonuclease XhoII in the NPTII coding sequence. This mutation was found to be present in the CaMV-NPTII gene of Fromm et al., 1986 and was repaired in the following manner. The plasmid pSUP2021 (Simon et al, 1983) is approximately 10kb in size and includes a complete copy of the transposon Tn5. Digestion of this plasmid with Pst 1 and Sma 1 gives a 788bp fragment that extends from position 1730 to 2518 within Tn5 (Beck et al., 1982). This fragment was isolated and restricted with Sph 1 (giving fragments of 352 and 436 bp) or XhoII (giving fragments of 120, 246, 394 and 28 bp), and is therefore "wild-type" with respect to the mutation at position 2096. The Pst 1/Sma 1 fragment was subcloned into Pst 1/Sma 1 cut pUC19 to give pTn5sub. This was then digested with Sma 1 and ligated with 8mer phosphorylated Bam H1 linkers. A clone in which the Sma 1 site had been converted to a Bam H1 site (pTn5subA) was then digested with Sph 1 and Bam H1 and the 436bp fragment (from position 2082 to 2518) isolated. This was used in a tripartite ligation with the 542 bp Bam H1/Sph 1 fragment from pCaMVNeo (positions 1540 to 2082) and Bam

H1 digested pUC19. Recombinants were restricted with Bam H1 and Sph 1 to ensure that they contained both the 436 and 542 Bam H1/Sph 1 fragments, and Xho II to confirm that the site at position 2096 had been restored. This construct has a Bam H1 fragment which contains the NPTII gene coding sequence which is essentially identical to the Bam H1 fragment used by Fromm et al., (1986) to make pCaMVNeo, except that the mutation has been corrected. This construct was designated pneoNeo. The Bam H1 insert of pneoNeo containing the NPT11 coding sequence was then isolated and religated with the large (approx. 3 kb) fragment isolated from Bam H1 restricted pCaMVNeo, this fragment containing the vector plus CaMV promoter and nopaline synthase gene 3' termination sequence. Recombinants were checked against pCaMVNeo for the correct orientation using both Pvu II (2 sites) or Eco R1/Sph 1 (both unique), giving pCaMVneoNeo. This was again checked for the correct number of Xho II sites.

The Hind III fragment from pCaMVneoNeo containing the restored plant-expressed kanamycin resistance gene was cloned into the Hind III site of pSCV1 to give the plasmid pSCV1.2. pSCV1.2 was partially digested with HindIII and the linear 10.2kb product isolated. This was dephosphorylated with calf intestinal alkaline phosphatase and ligated with a 2.8kb Hind III DNA fragment containing a plant expressed ß-glucuronidase gene (CaMV-GUS INT gene) isolated from the plasmid pGUS INT which has been described by Vancanneyt et al., 1990.

A map of the T-DNA in the resultant construct (pSCV1.6), indicating the orientation of the genes and the region of DNA for transfer to plants are shown in Figure 2.

In Figure 2 the abbreviations given in the map have the following meanings: B = Bam H1; Bg = Bgl II; C = Cla

1; E = Eco R1; EV = Eco RV; H = Hind III; K = Kpn 1; P = Pst 1; S = Sac 1; Sm = Sma 1; Sp = Sph 1; X = Xba 1; Xh = Xho 1; OD = Over-drive (T-DNA transfer enhancer)

Introduction of the binary plasmid vector pSCV1.6 into the disarmed A. tumefaciens strain

Cells of Agrobacterium tumefaciens strain EHA101A were transformed by electroporation using a Biorad Gene Pulser as described by Wen-jun and Forde (1989).

Preparation of Agrobacterium inoculum

10 ml overnight liquid cultures of Agrobacterium tumefaciens strain EHA101A containing the binary plasmid pSCV1.6 were grown on liquid YEB medium (tryptone 5 g l^{-1}, yeast extract 1 g l^{-1}, beef extract 5 g l^{-1}, magnesium sulphate 0.46 g l^{-1}, pH 7.2 and sucrose 5 g l^{-1} added after autoclaving) containing 50 mgl^{-1} chloramphenicol, 25 mg l^{-1} neomycin and 15 mg l^{-1} gentamicin at 28°C with vigorous shaking. The cells were harvested by centrifugation at 6000g for 10 minutes and resuspended in 10ml liquid KM medium. A control strain of Agrobacterium tumefaciens (EHA101A) lacking the binary plasmid pSCV1.6 was grown under similar conditions except that the gentamycin was excluded from the growth medium. Cell titres for each strain were estimated by plating on YEB plates (as for liquid YEB medium but containing 1.5% w/v bacto-agar) containing the relevent antibiotics for each strain as described above.

Transformation of intact shoots in air-lift fermenters

After inoculation with plant material and operation of the air-lift fermenters for 4-5 days, the liquid medium was removed and replaced with fresh liquid KM medium. The vessel was inoculated with the Agrobacterium

strain to a final titre of 2 x 10^7 CFU (colony forming units) ml^{-1} and operation of the air-lift fermenter continued. If required, additional bacteria were inoculated into the fermenter at various intervals, either accompanied with medium replacement of without medium replacement. The degree of transformation of the shoot material was determined by histochemical ß-glucuronidase assay as described below. Once sufficient shoot material of the required degree had been obtained, the medium was removed and the plant material rinsed twice in fresh liquid KM medium. Fresh liquid KM medium containing 600 mg l^{-1} augmentin was added to the fermenter and operation continued for 4 hrs. The medium was then replaced with fresh KM medium containing 300 mg l^{-1} augmentin and operation continued overnight prior to harvest of the transformed shoot material and subsequent plating onto regeneration medium.

Regeneration of putative transgenic shoots

After transformation in liquid culture, the resultant shoots were harvested and dissected. The resultant leaf, petiole or stem explants were then transferred to clonal regeneration medium (750 mg l^{-1} KNO_3, 250mg l^{-1} $MgSO_4.7H_2O$, 250 mg l^{-1} $NH_4H_2PO_4$, 100mg l^{-1} $CaCl_2.2H_2O$, 20 g l^{-1} sucrose, 600 mg l^{-1} 2-[N-morpholino]ethanesulphonic acid (MES), half-strength Murashige and Skoog basal salt micronutrient solution (Sigma catalogue number M0529), vitamins as described by Morel and Wetmore (1951), 500 mg l^{-1} glutamine, 50 mg l^{-1} ascorbic acid, 0.1 to 1 (eg 1) mg l^{-1} CPPU, 0.465 mg l^{-1} NAA, pH adjusted to pH 5.5 with KOH, 3 g l^{-1} phytagel) containing 300 mg l^{-1} augmentin and either 0, 50 or 100 mg l^{-1} paromomycin or 25 mg l^{-1} G418.

The explants were incubated in the dark at 23°C for 4 weeks with subculture to fresh medium after 2 weeks

and at the end of the period of incubation in the dark. The cultures were then transferred to continuous light (40 µmol m^{-2} s^{-1}) and incubated at 23°C. The cultures were then subcultured every two weeks onto fresh clonal shoot induction medium until significant numbers of shoot primordia were visible. The explants were subcultured onto clonal shoot elongation medium (as clonal shoot induction medium) but with the CPPU ommitted, the NAA concentration adjusted to 0.112 mg l^{-1} and containing 1.16 mg l^{-1} BAP and incubated at 23°C under continuous light (40 µmol $m^{-2}s^{-1}$).

Selection, multiplication and rooting of putative genetically modified shoots

When suitable numbers of regenerating shoots more than 1mm long were present on the explants, the explants were transferred to an air-lift fermenter containing the liquid KM micropropagation medium described above, 300 mg l^{-1} augmentin and 30-120 mg l^{-1} paromomycin, generally 50-60 mg l^{-1}. After a suitable period, generally 5-20 days depending on the concentration of paromomycin used, putative genetically modified shoots are identified by their healthy green appearance and rapid growth and extension in comparison to the majority of non-GM shoots which become brown and necrotic.

The putative genetically modified shoots were then transferred to fresh liquid KM micropropagation medium (with or without paromomycin) and multiplied for a further 1 month, each individual original shoot now forming a large mass of branching shoots. These shoots were then rooted by transfer to rooting medium (as clonal shoot multiplication medium i.e. semi-solid KM medium but with the BAP omitted and containing 0.2 mg l^{-1} IBA) and incubation for 24h at 23°C using 16 hour day illumination regime of 50-70 µmol $m^{-1}s^{-1}$. Additional

multiplication steps, either in liquid culture or on solid culture may be conducted as required prior to rooting of shoots.

Following the root-induction step, shoots with developing roots were transferred to a sterile peat pellet (Jiffy Products (UK) Limited, 14/16 Commercial Road, March, Cambridge, UK) in a Magenta pot (Sigma) for root establishment.

When actively growing roots were visible growing through the peat pellet, the plant was transferred to an approximately 7.5 cm (3 inch) square plant pot filled with coco-peat. The plants were placed inside a mist propagator and slowly hardened off by reducing the humidity over a period of a week. After three to four weeks, the plants were transferred to approximately 17.5 cm (7 inch) pots and placed in a glasshouse facility. The plants were grown under natural daylight and were watered daily.

Alternatively, shoots harvested from fermenters were set for rooting ex-vitro in 50:50 mix of coco-peat and perlite in a mist propagator at 23°C using 16 hour day illumination regime of 30-40 mmol $m^{-1}s^{-1}$ and cultivated for 1-2 weeks. The humidity was gradually reduced to ambient and the light intensity gradually increased to 100 mmol $m^{-1}s^{-1}$ over a period of several weeks. Rooted shoots were then potted-on as previously described.

Biochemical and genetic analysis of the genetically modified Eucalyptus plants

Histochemical ß-glucuronidase (GUS) assays

Histochemical GUS assays were performed Eucalyptus clones as described by Draper et al. (1988). Leaf explants were transferred to a petri dish containing fixation solution (100 ml double distilled water

containing 750 ml 40% formaldehyde, 2 ml 0.5 M MES and 5.46 g l^{-1} Mannitol). The petri dish was placed in a vacuum desiccator and the vessel was evacuated several times until all of the explants were infiltrated with the fixation solution. The explants were incubated for 45 minutes at room temperature and then washed twice in 50mM sodium phosphate buffer (pH 7.0). The explants were then transferred into a 2mM 5-bromo-4-chloro-3-indoyl glucuronide (X-GLUC) solution made up in 50mM sodium phosphate buffer (pH 7.0). The X-GLUC solution was vacuum infiltrated into the explants, the dish sealed with Nescofilm and then incubated at 37°C overnight. The reaction was stopped by transferring the explants to 70% ethanol. GUS activity could be detected by the presence of an insoluble blue stain.

Analysis of genetically manipulated Eucalyptus plants using the polymerase chain reaction (PCR) for the presence of the T-DNA and for absence of the Agrobacterium tumefaciens strain used in their production

PCR reactions were conducted using a Perkin-Elmer Cetus DNA Thermal Cycler (Perkin-Elmer, Beaconsfield, Bucks. UK). The reaction consisted of 1x reaction buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% w/v gelatin), 200 µM each dNTP, 1.0 µM of each primer, 2.5 units of Amplitaq DNA polymerase (Perkin-Elmer) and 0.5 µg genomic DNA isolated from genetically manipulated shoots as described in Keil & Griffin (1994). Control reactions containing genomic DNA from plants known to be free of Agrobacterium tumefaciens or containing approximately 10 ng DNA isolated from Agrobacterium tumefaciens EHA101A [pEHA101, pSCV1.6] were also conducted. Reaction conditions used were 29 cycles of 1 min at 94°C, 1 min annealing and 1 min at 72°C and one

cycle of 1 min at 94°C, 1 min annealing and 2 min at 72°C. Samples of each reaction were electrophoresed on a 2% agarose gel and visualised under UV light. Primers for the detection of the following gene sequences and the annealing temperatures used in the reactions were as described below:

NPTII gene (using the TN5 numbering system described by Beck et al., (1982) Gene 19, 327-336).

PRIMER 1: 5' (24) CGCAGGTTCTCCGGCCGCTTGGGTGG (50) 3'

PRIMER 2: 5' (277) AGCAGCCAGTCCCTTCCCGCTTCAG (253) 3'

Annealing temperature 50°C

ros gene of Agrobacterium tumefaciens chromosome (using the numbering system of Cooley et al., (1991), J. Bacteriology 173, 2608-2616); primers by Matzk and Schiemann (Poster No. S7-23, 8th International Congress of Plant Tissue and Cell Culture, Firenze, Italy, June 12-17 1994).

PRIMER 1: 5' (142) CGCGGGCTACAAGTTGAATC (161) 3'

PRIMER 2: 5' (714) GACCGAGACCCATTTCCTTG (695) 3'

Annealing temperature 60°C

Vir G gene of the virulence gene of Agrobacterium tumefaciens Ti plasmid (using the numbering system of Chen et al., (1991), Mol Gen Genet 230, 302-309); primers designed by Matzk and Schiemann (Poster No. S7-23, 8th International Congress of Plant Tissue and Cell Culture, Firenze, Italy, June 12-17 1994).

PRIMER 1: 5' (370) GCCGACAGCACCCAGTTCAC (389)
PRIMER 2: 5' (749) GCCGTAAGTTTCACCTCACC (730)

Annealing temperature 60°C

The band diagnostic for the NPT11 gene sequence acts as a positive control reaction in that this product should be detected in both in the genetically modified plants and in plants infected with the Agrobacterium strain used for their production (EHA101A [pEHA101, pSCV1.6]). Presence of the bands diagnostic for the ros and virG genes are indicative that the plants are still infected with the Agrobacterium strain used in their production (EHA101A [pEHA101, pSCV1.6]).

Detection of contaminants in genetically manipulated plants using microbiological assay

Genetically manipulated plants were homogenised in sterile mortar and pestles and the resultant homogenate was transferred aseptically to shake flasks containing sterile YEB medium (as descibed previously) without antibiotics. The flasks were incubated at 29°C with vigorous shaking for a minimum of 5 days. Lack of microbial growth is indicative that the genetically manipulated plants are free of Agrobacterium.

Detection of genes transferred to transgenic Eucalyptus plants by Southern blotting and hybridisation

DNA extraction was carried out as described by Keil and Griffin (1994). 10 micrograms of DNA isolated from transformed Eucalyptus plants were digested with HindIII in the appropriate restriction buffers. To aid the digestion of DNA, casein was added to the restriction mixture at a final concentration of 0.1 mg/ml (Drayer and Schulte-Holthausen, 1991). The restrictions were carried out at 37°C overnight. Electrophoresis of the

samples, Southern blotting and hybridisation were performed as described by Sambrook et al. (1989). The plasmid pJIT65 (Guerineau, 1990) was digested with Eco RV and the plasmid pCaMV digested with Bam H1. The resulting restriction fragments were separated by electrophoresis on a 1.5% agarose gel (Sambrook et al., 1989). A 2kb (approximately) DNA fragment containing part of the coding sequence of the GUS gene and the Cauliflower Mosaic Virus 35S gene terminator region and a 1.0 kb (approximately) DNA fragment containing the NPT2 coding sequence were eluted from the gel by the method of Heery et al. (1990). The eluted fragments were radiolabelled by the method of Feinberg and Vogelstein (1983), using the random primer labelling kit supplied by Boehringer Manheim and used as hybridisation probes.

TRANSFORMATION OF E. GRANDIS AND E. GRANDIS HYBRID CLONES IN LIQUID MEDIUM AND REGENERATION OF SHOOTS ON SEMI-SOLID MEDIUM

Method

Air-lift fermenters were set up, inoculated with E. grandis and E. grandis hybrid plant material and operated as described above. The vessels were then inoculated with the Agrobacterium strain containing plasmid pSCV1.6, and the plant material and the Agrobacteria were co-cultivated for three weeks as described above. Control fermenters were inoculated with the Agrobacterium strain lacking pSCV1.6.

Three weeks after inoculation, the liquid medium was discarded from the fermenters and the shoot masses rinsed twice in 500 ml sterile liquid KM micropropagation medium and then washed twice in 1l liquid KM medium containing 600 mg l^{-1} augmentin for 3 hours per wash with the fermenter in operation. 2 l of

fresh liquid KM medium containing 300 mg l^{-1} augmentin was added to each fermenter and operation of the fermenters continued for a further 24 hrs. Shoot masses were harvested and dissected. Leaf, petiole and stem explants plated onto semi-solid regeneration medium containing 300 mg l^{-1} augmentin and either 0, 50 or 100 mg l^{-1} paromomycin or 25 mg l^{-1} G418 as previously described.

After 9 to 17 weeks on semi-solid regeneration medium depending on the selection conditions used, explants with regenerating shoots were transferred air-lift fermenters for selection on paromomycin as previously described. Healthy shoots which survived the liquid selection step were transferred to fresh KM medium containing 300 mg l^{-1} augmentin and grown on for 3-4 weeks prior to harvest and subsequent roots as previously described.

Results

Slight turbidity of the medium in the air-lift fermenters inoculated with both strains of Agrobacterium became noticable approximately 24 hrs after inocation and continued to increase gradually over a further period of approximately 24 hrs and then stabilised. Titres of Agrobacterium cells as determined by plating showed that numbers increased from approximately 2 x 10^7 CFU ml^{-1} at inoculation to a maximum of approximately 3 x 10^9 CFU ml^{-1} 48 hrs after inoculation. Agrobacterium cells titres then gradually decreased over the following 3 weeks to give final cell densities in the range 3-6 x 10^8 CFU ml $^{-1}$. This slow-down and cessation of growth of the Agrobacterium strains was accompanied by a reduction in the pH of the growth medium in the fermenters. The pH had reduced from 5.6 at inoculation of the Agrobacterium strains to pH 5.2 at 24 hrs after

inoculation and pH 4.5 at 48 hrs after inoculation. The pH remained at approximately 4.5 for the remaining three weeks of operation. The pH of the medium in a fermenter containing shoots operated in an identical manner but not inoculated with Agrobacterium also showed a similar decline in pH.

1 week after inoculation of the fermenters with the Agrobacterium strains, the amount of plant material had increased significantly. Shoot samples were taken and histochemically stained for ß-glucuronidase activity. ß-glucuronidase activity was apparent in shoots co-cultivated with the Agrobacterium strain containing the plasmid pSCV1.6 as numerous areas of blue-stained tissue each consisting of groups of cells in leaves, petioles and stems. Both the number of blue staining areas and the size of the individual areas were greatest in the older tissues, although blue-staining was also present in younger tissues .

2 weeks after inoculation of the fermenters with the Agrobacterium strains, the plant material was continuing to grow rapidly. Shoot masses were harvested and stained for ß-glucuronidase activity. ß-glucuronidase activity was detected in shoots co-cultivated with the Agrobacterium strain containing the plasmid pSCV1.6. Both the size and extent of the areas giving a positive staining reaction had increased in the older tissues with numerous small and large areas giving a positive reaction. The maximum size of the areas of tissue giving a positive staining reaction was largest for the older tissues and smallest for the youngest tissues. Small areas of blue staining tissues were present in new growth, indicating that transformation of these tissues had occurred after the initial burst of growth by the Agrobacterium strain.

3 weeks after inoculation, the shoot masses almost

completely filled the vessels and the rate of growth of the plant tissues was decreasing. The patern of transformation was similar to that seen at two weeks after inoculation, except that the size and extent of the areas staining for ß-glucuronidase activity had further increased, particularly in younger tissues where numerous areas of transformed tissues, many of which were quite large were observed. The degree of transformation of the tissues was very large. For instance, more than 50% of the tissues in some leaves appeared to be transformed. Hand dissection of the tissues showed that some of the transformed areas consisted of multiple cell types. For example, epidermal, pallisade and mesophyl cells within some single contiguous areas were all found to stain for ß-glucuronidase activity.

No GUS activity was detected in the air-lift fermenters inoculated with the Agrobacterium strain lacking pSCV1.6 in any of the assays conducted.

The period required for explants harvested from the fermenters to produce shoots of 1mm larger and on semi-solid regeneration medium under the different selection conditions are shown in Table 1. The number of explants (expressed as a percentage of the number originally plated on regeneration medium and assessed at the culture period indicated) subsequently producing shoots of 1mm or larger and deemed suitable for transfer to liquid culture are also shown. Figures in brackets are for explants transformed with the control Agrobacterium strain lacking pSCV1.6 and regenerated and selected under identical conditions to the material co-cultivated with the strain containing pSCV1.6.

TABLE 1

selection treatment during regeneration	culture period (weeks) on regeneration medium required to produce shoots 1mm or larger	% explants producing shoots (of total plated on regeneration medium) at culture period indicated
0 (no selection)	9	73 (68)
50 mg ml^{-1} paromomycin	11	26 (12)
100 mg ml^{-1} paromomycin	15	14 (6)
25 mg ml^{-1} G418	17	30 (24)

Explants producing shoots of 1mm or larger were harvested from regeneration medium when deemed suitable for transfer to liquid culture at the times indicated in Table 1. The percentages of explants (of those originally plated onto semi-solid regeneration medium) which produced shoots on the different selection regimes on semi-solid regeneration medium and subsequently possessing shoots of healthy appearance after 5 and 18 days selection on 60 mg ml^{-1} paromomycin in liquid culture are shown in Table 2. Figures in brackets are for explants transformed with the control Agrobacterium strain lacking pSCV1.6 and regenerated and selected under identical conditions to the material co-cultivated with the strain containing pSCV1.6.

TABLE 2

selection treatment during regeneration	number of shoots (expressed as % of explants planted on regeneration medium) of healthy appearance after selection on 60 mg ml^{-1} paromomycin in liquid culture after		surviving shoot clusters giving +ve GUS reaction (expressed as % of explants plated)
	5 days	18 days	
0 (no selection)	8 (6)	1 (0)	1
50 mg ml^{-1} paromomycin	17 (4)	6 (0)	5
100 mg ml^{-1} paromomycin	12 (4)	5 (0)	5
25 mg ml^{-1} G418	19 (8)	2 (0)	2

All the shoot clusters surviving 18 days selection on 60 mg ml^{-1} paromomycin in liquid culture gave a positive reaction when tested for the presence of the NPT11 gene using the polymerase chain reaction. Negative PCR reactions were obtained for the ros and Vir G genes, indicating that the shoots produced were free of residual Agrobacterium cells. Microbiological assay for the presence of surviving Agrobacterium cells also gave a negative result. All of the surviving shoots contained at least one copy of the NPT11 and B-glucuronidase genes as detected by Southern blotting. Transformed shoots which were rooted and grown on the greenhouse were of normal appearance and were indistinguishable for untransformed controls.

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C L A I M S

1. A process for producing genetically modified plant material comprising one or more stably incorporated DNA sequence(s) of interest, which process comprises subjecting plant material that consists of or comprises shoots, shoot tips or apexes, rooted shoots or seedlings, to bacterium-mediated transfer of the DNA sequence(s), the bacterium-mediated DNA transfer being carried out in an oxygenated liquid culture medium in which the plant material is cultivated, the plant material being submerged in the liquid medium.

2. A process as claimed in claim 1, wherein the bacterium-mediated transfer of the DNA sequences is Agrobacterium-mediated transfer.

3. A process as claimed in claim 1 or claim 2, wherein the liquid medium is maintained such that plant growth proceeds normally but growth of the bacterium is restrained.

4. A process as claimed in any one of claims 1 to 3, wherein the liquid medium is maintained such that transfer of the DNA of interest from the bacterium to the plant material is stimulated.

5. A process as claimed in any one of claims 1 to 3, wherein the pH of the liquid medium is maintained at a level at which plant growth proceeds normally but growth of the bacterium is restrained.

6. A process as claimed in claim 1, claim 2 or claim 3, wherein the pH of the liquid medium is maintained at a level at which transfer of the DNA of interest from the bacterium to the plant material is stimulated.

7. A process as claimed in any one of claims 1 to 6, wherein the pH of the medium is maintained within the range of from 4.0 to 5.4.

8. A process as claimed in any one of claims 1 to 7, wherein oxygen is provided by passing air or oxygen through the medium, by mechanical agitation means, by means of shaking the medium, by means of illuminating the medium, or by any two or more of said means.

9. A process as claimed in any one of claims 1 to 8, wherein the liquid medium is agitated.

10. A process as claimed in claim 9, wherein the culture medium is agitated and oxygen is provided by passing oxygen or air through the medium.

11. A process as claimed in any of one claims 1 to 10, wherein the shoots, shoot tips or apexes, rooted shoots or seedlings are free to move in the liquid medium.

12. A process as claimed in claim 11, wherein the shoots, shoot tips or apexes, rooted shoots or seedlings tumble in the liquid medium.

13. A process as claimed in any one of claims 1 to 12, wherein the plant material is obtained from a cultivar, clone or seed.

14. A process as claimed in any one of claims 1 to 13, wherein the plant material is from an annual, biennial or perennial plant.

15. A process as claimed in any one of claims 1 to 14, where the plant material is from a monocotyledonous or dicotyledonous plant.

16. A process as claimed in any one of claims 1 to 15, where the plant material is from a herbaceous plant.

17. A process as claimed in any one of claims 1 to 13, wherein the plant material is from a woody plant.

18. A process as claimed in claim 17, wherein the woody plant is a gymnosperm or dicotyledonous or monocotyledonous angiosperm used for wood pulp, for fuel or for timber; a tree, shrub or bush that produces fruit or nuts; a tree or shrub from which a commercially useful product other than a fruit or nut is obtained; or

an ornamental tree or shrub.

19. A process as claimed in claim 17, wherein the woody plant is of a sclerophyllous species.

20. A process as claimed in claim 17, where the woody plant is a Rhododendron, Azalea or Kalmia (Ericaceae); an Olea (Oleaceae); or an Australian Acacia.

21. A process as claimed in claim 17, wherein the woody plant is a Malus (apple); Pyrus, Prunus or Rosa (Rosaceae); Forsythia or Syringia (Oleaceae).

22. A process as claimed in claim 17, wherein the woody plant is a tree.

23. A process as claimed in claim 17, wherein a woody plant is a Pinus, Picea, Abies, Pseudotsuga, Chaemaecypris, Taxus, Populus, Acacia, Leucaena, Melia, Gmelina, Liquidambar, Betula, Hevea, Tectona, Alnus, Grevillea, Paulonia, Cedrela, Coffea, Citrus, Phoenix, Juglans or Elaeis.

24. A process as claimed in claim 17, wherein the woody plant is a eucalypt.

25. A process as claimed in claim 24, wherein the woody plant is a eucalypt of the sub-genus Eucalyptus Symphyomyrtus, Eucalyptus Corymbia, or Eucalyptus Monocalyptus.

26. A process as claimed in claim 25, wherein the eucalypt is E. grandis, E. globulus, E. nitens, E. dunnii, E. saligna, E. camaldulensis, E. urophylla, E. tereticornis or a hybrid thereof, or E. regnans, E. citriodora, E. fraxinoides E. maculata or a hybrid thereof.

27. A process as claimed in any one of claims 1 to 26, wherein shoot formation is induced in the genetically modified plant material in the presence of an agent capable of inducing the formation of shoots.

28. A process as claimed in claim 27, wherein the shoot inducing agent is a cytokinin.

29. A process as claimed in claim 27, wherein the shoot inducing agent is 6-benzylaminopurine or N-(2-chloro-4-pyridyl)-N'-phenylurea.

30. A process as claimed in any one of claims 1 to 29, wherein the genetically modified plant material has a selectable property, attribute or characteristic that enables selection, and the resulting genetically modified plant material is selected.

31. A process as claimed in claim 30, wherein the genetically modified plant material has a selectable property, attribute or characteristic that enables selection using a selective agent, and the resulting genetically modified shoots are selected on a semi-solid medium that comprises the selective agent and/or in an oxygenated liquid culture medium comprises the selective agent, the shoots being submerged in the liquid medium.

32. A process as claimed in claim 31, wherein the selectable property is resistance to an antibiotic, herbicide or other selective agent.

33. A process as claimed in any one of claims 27 to 32, wherein the culture medium comprises an antibiotic.

34. A process as claimed in any one of claims 31 to 33, wherein the selection in a liquid culture medium is carried out before and/or after selection on a semi-solid medium.

35. A process as claimed in any one of claims 31 to 34, wherein shoot induction and, if desired, simultaneous selection under non-stringent conditions is carried out on a semi-solid medium, then selection under stringent conditions is carried out in submerged oxygenated liquid culture.

36. A process as claimed in claim 30, wherein the genetically modified plant material has a selectable property, characteristic or attribute that enables selection by the detection of an introduced nucleic acid

sequence or of the product of an introduced nucleic acid sequence.

37. A process as claimed in claim 36, wherein the product of an introduced nucleic acid sequence is an enzyme, a detectable pigment or a detectable fluorescent moiety.

38. A process as claimed in any one of claims 1 to 37, wherein the resulting genetically modified plant material is micropropagated in an oxygenated liquid culture medium, the plant material being submerged in the liquid medium.

39. Genetically modified plant material obtained according to a process as claimed in any one of claims 1 to 38.

40. A genetically modified plant obtained from genetically modified plant material as claimed in claim 39.

41. A genetically modified plant obtained by propagation of a genetically modified plant as claimed in claim 40 or of genetically modified plant material as claimed in claim 39.

42. A product obtained from a plant as claimed in claim 40 or claim 41.

Fig.1.

MAP OF pSCV1

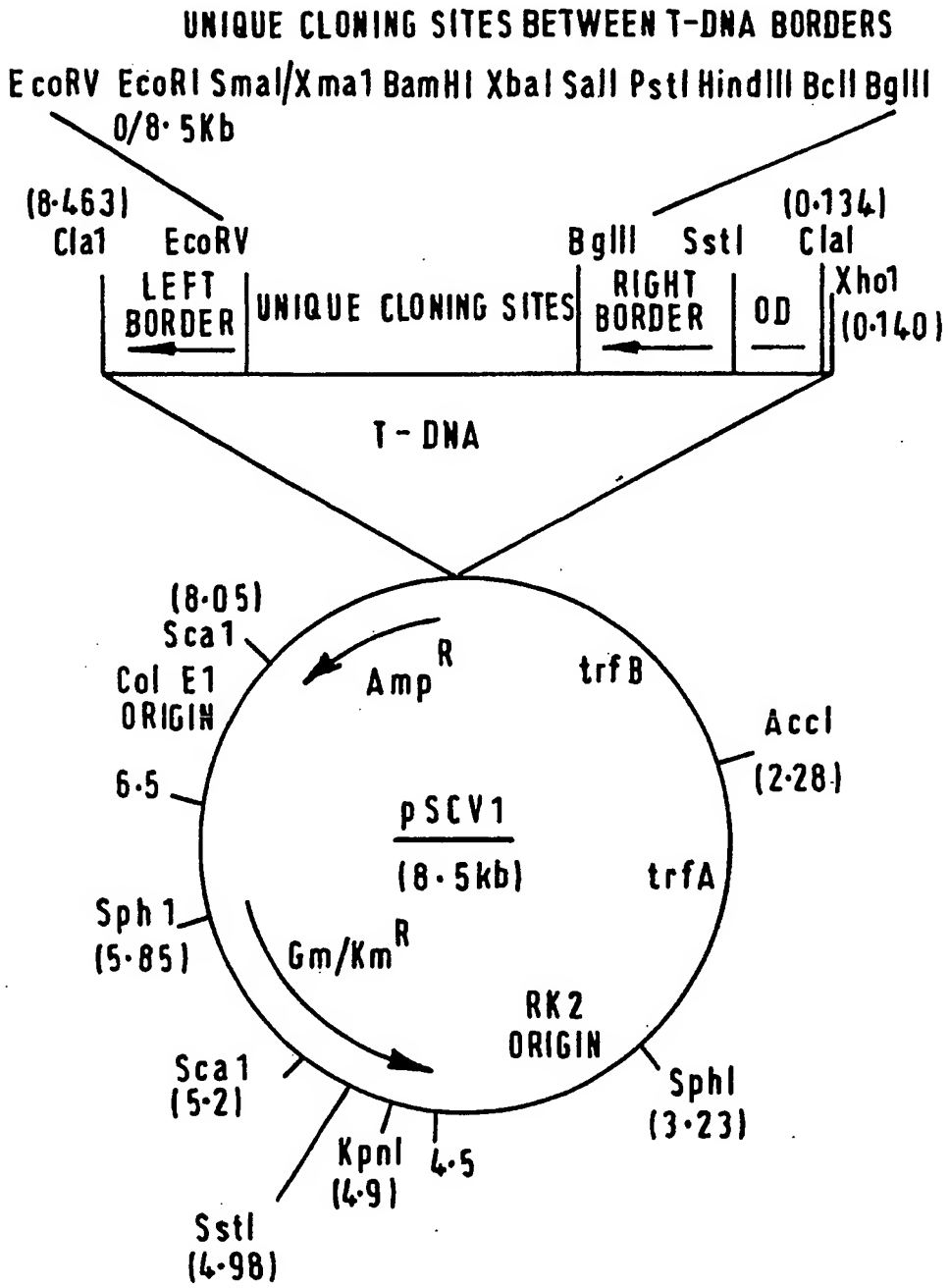


Fig.2.

T-DNA OF pSCV1-6

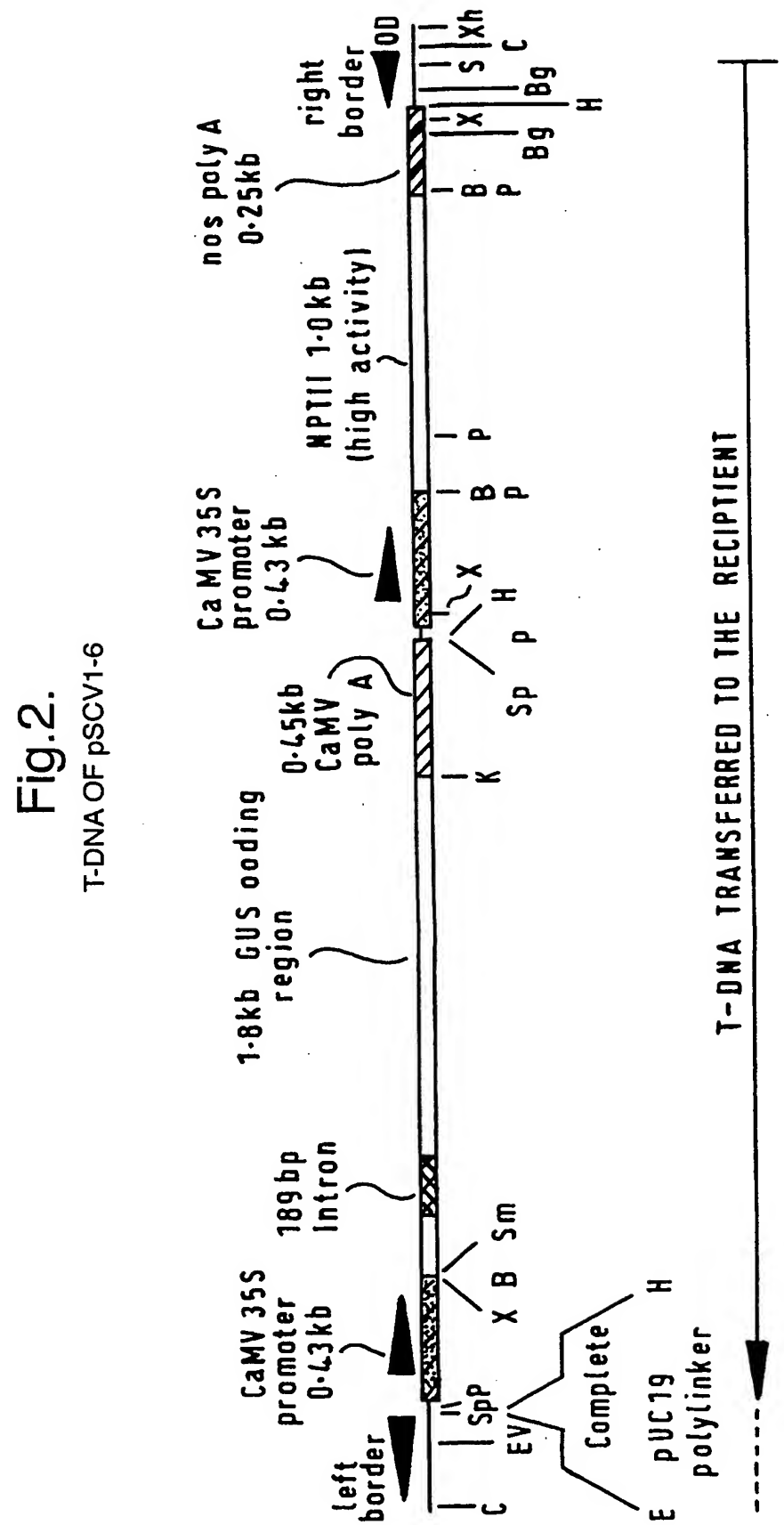
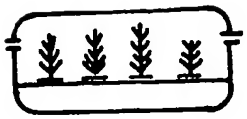
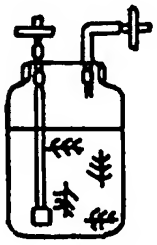


Fig.3.

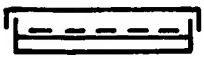
STEP 1



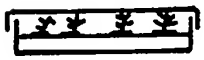
STEP 2



STEP 3

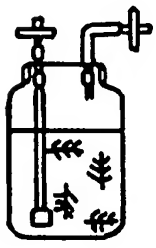


STEP 4

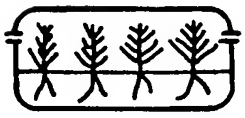


STEP 5

STEP 6



STEP 7



INTERNATIONAL SEARCH REPORT

Interr. nal Application No
PCT/EP 98/03707

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GB 2 211 204 A (OJI PAPER CO) 28 June 1989 * see esp. p.8/9; examples * ---	1,2,8,9, 11-23, 27-34, 36,37, 39-41
X	DATABASE WPI Section Ch, Week 9624 Derwent Publications Ltd., London, GB; Class A01,Page 6, AN 96-233218 XP002030466 & JP 08 089113 A (OJI PAPER CO.) , 9 April 1996 see abstract --- -/--	1-9, 11-33, 36-42

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 October 1998	09/11/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Kania, T

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INTERNATIONAL SEARCH REPORT

Intern nal Application No
PCT/EP 98/03707

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	VERNADE D ET AL: "GLYCINE BETAINE ALLOWS ENHANCED INDUCTION OF THE AGROBACTERIUM TUMEFACIENS VIR GENES BY ACETOSYRINGONE AT LOW PH" JOURNAL OF BACTERIOLOGY, vol. 170, no. 12, December 1988, pages 5822-5829, XP002067609 * see esp. p.5827 last par. - p.5828 *	4-7
A	GB 2 298 205 A (SHELL INT RESEARCH) 28 August 1996 cited in the application see the whole document	1-42
A	ADAM S ET AL: "TRANSFORMATION GENETIQUE ET REGENERATION DE BOURGEONS TRANSGENIQUES CHEZ EUCALYPTUS GUNNII" BIOTECHNOLOGIES APPLIQUEES AUX ARBRES FORESTIERS,1991, pages 81-110, XP000671376 see the whole document	1-42

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/EP 98/03707

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB 2211204 A	28-06-1989	FR 2621780 A	21-04-1989
		JP 2138966 A	28-05-1990
GB 2298205 A	28-08-1996	AU 4940296 A	04-09-1996
		WO 9625504 A	22-08-1996
		EP 0808372 A	26-11-1997

Susan Olson

From: Joseph Mallon
Sent: Monday, March 29, 2004 5:17 PM
To: Susan Olson
Subject: ASMEX.291A/320A/328A/333A/367A

Susan,

Please check these cases to see whether we've submitted US 5,227,329 and US 5,389,570 in IDS's for these cases. If not, pls prepare the IDS's.